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Expression of cellular prion-related protein by murine Langerhans cells and keratinocytes

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Abstract

Transmissible spongiform encephalopathies are characterized by the accumulation of a proteinase-resistant isoform of the cellular prion-related protein (PrP^c) within the central nervous system (CNS). The accumulation of scrapie-associated PrP (PrP^{Sc}) within cells of the lymphoreticular system prior to its accumulation in the CNS is regarded as important for the development of neurological diseases after peripheral inoculation. Little, however, is known as to which cells are the targets for peripheral inoculation. Here, the presence of PrP^c on murine Langerhans cells (LC), dendritic cells in the skin and mucosa, and keratinocytes (KC) is demonstrated by immunohistochemical staining, Western-blotting and FACS analysis. The expression of PrP^c mRNA in freshly purified LC and KC was also detected by reverse transcriptase-polymerase chain reaction. The expression of PrP^c on LC was slightly increased during culture. These data suggest that LC and KC may be the targets for peripheral infection with prions.

Keywords: Prion-related protein; Langerhans cells; Keratinocytes; Peripheral inoculation; Lymphoreticular system

1. Introduction

Prions are infectious particles causing transmissible spongiform encephalopathies (TSEs), including scrapie, kuru and Creutzfeldt–Jakob

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disease (CJD). They consist, at least in part, of an abnormal isoform (scrapie-associated PrP, PrP^{Sc}) of the ubiquitous cellular prion-related protein (PrP^{c}). Conformational differences between PrP^{c} and PrP^{Sc} are evident from increased β-sheet content and proteinase resistance in PrP^{Sc} [1–3]. PrP^{Sc} can be detected in various organs other than the central nervous system (CNS). Especially, the accumulation of PrP^{Sc} within cells of the lymphoreticular system (LRS) prior to its accumulation in the CNS is regarded as important for the development of neurological diseases after peripheral inoculation [4–6]. On the other hand, the existence of PrP^{c}, a potential reservoir of PrP^{Sc}, is regarded as important for the onset of TSEs because PrP^{c} knockout mice are resistant to infection with prions [7] and hamster PrP^{c} transgenic mice developed neurological disease after hamster-specific scrapie inoculation, whereas wild-type mice did not [8]. Various tissues other than the CNS, such as spleen, lung, heart, kidney and uterus, have been proved to express PrP^{c}, although the strength of its expression is varied in different tissues [9–15]. It is reported that brain expresses PrP^{c} most strongly at both mRNA and protein levels. On the contrary, neither PrP^{c} protein nor mRNA is detected in liver, indicating tissue-specific function of PrP^{c}. As for the skin, recently human and mice keratinocytes (KC), the main cell type of the epidermis, were found to express PrP^{c} [16,17].

Langerhans cells (LC), dendritic cells in the skin and mucosa, are connected with peripheral neurons in the epidermis [18] and migrate to peripheral lymph nodes upon activation [19], which suggests the possibility that LC are involved in the transmission of peripherally inoculated PrP^{Sc} to the LRS and CNS. However, to the best of our knowledge, LC have not yet been studied for PrP^{c} expression and its possible involvement in prion diseases. Therefore, we investigated the expression of PrP^{c} on murine LC and KC.

2. Materials and methods

2.1. Animals

BALB/c female mice and C57BL/6 female mice (wild type) were obtained from Japan SLC Co. (Hamamatsu, Japan) and were maintained until 8–12 weeks of age under specific-pathogen-free conditions in our animal facilities. PrP^{c} knockout mice (*Prnp* $-/-$) were generated by homologous recombination as described previously [20,21].

2.2. Reagents

Mouse anti-PrP^{c} polyclonal antibodies were generated by immunizing PrP^{c} knockout mice with 10% brain homogenates or 2×10^{7} thymocytes from wild-type mice (C57BL/6) as described previously [21]. FITC-mouse anti-mouse I-A^{d} (clone AMS-32.1) and FITC-mouse IgG 2b (PharMingen, San Diego, CA) were used for flow cytometry. FITC-streptavidin and PE-streptavidin (PharMingen, San Diego, CA) were used as second staining reagents.

2.3. Cell preparation

Murine LC were enriched using the panning method as previously described [22] with slight modifications [23], and the purity of LC estimated in the first several experiments was over 95%. Briefly, murine truncal skin was treated with dispase (3000 U/ml, Godo Shusei, Tokyo, Japan) for 3 h for BALB/c mice and for 8 h for C57BL/6 mice at 37 °C. Epidermis was separated from dermis and incubated with 0.025% DNase (Sigma Chemical Co., St. Louis, MO) for 20 min at room temperature. Epidermal cell suspension was obtained by gently mixing and pipetting the epidermal sheets. The epidermal cell suspension was then treated with mouse anti-mouse I-A^{d} monoclonal antibody (Meiji Co., Tokyo, Japan) (1:600) for BALB/c mice or mouse anti-mouse I-$A^{b,k}$ monoclonal antibody (Meiji Co.) (1:600) for C57BL/6 mice for 45 min on ice. After washing, the cells were incubated for 45 min at 4 °C in plates which had been coated with goat anti-mouse IgG (Fc) (Cappel, Aurora, OH) (1:100). After washing out floating cells, adherent cells were collected and used as freshly isolated LC (fLC). The floating cells were virtually all KC, and were used as such in these experiments.

2.4. Cell culture

Freshly isolated LC (3×10^5 cells/well) were incubated in 96-well flat-bottom plates (Becton Dickinson Labware, Franklin Lakes, NJ) in RPMI 1640 medium supplemented with 10% FCS and antibiotics. After 36 h, cells were collected and used as cultured LC (cLC).

2.5. Flow cytometry

The surface phenotype of KC, fLC and cLC was identified using various monoclonal antibodies and a FACScan flow cytometer (Becton Dickinson). Briefly KC, fLC or cLC were incubated with antibodies (1:100) for 30 min at 4 °C, and then washed with PBS containing 1% FCS three times. Second reagents (1:100) were added as necessary and KC, fLC and cLC were incubated for 30 min at 4 °C, and then washed three times. Samples were then analyzed using the flow cytometer. Propidium iodide (Becton Dickinson, San Jose, CA) was added in order to exclude dead cells and mean fluorescent intensity (MFI) was determined for PrP^c.

2.6. Western blot analysis

Cells were lysed in 1% SDS buffer and denatured at 100 °C for 15 min. SDS-polyacrylamide gel electrophoresis and transfer to nitrocellulose membranes were performed under standard conditions [24]. Membranes were reacted with biotin-conjugated anti-mouse PrP^c followed by streptavidin-alkaline phosphatase (Oncogene Research Products, Cambridge, MA). After rinsing, Alkaline phosphatase (AP) Color Development Buffer and an AP conjugate substrate kit (Bio-Rad Lab. Hercules, CA) were used for color development.

2.7. Confocal laser scanning microscopic studies

To confirm that LC express PrP^c, we used a confocal laser scanning microscope as described previously [25]. Mice truncal skin was collected and snap-frozen in liquid nitrogen. Eight-micrometer cryostat sections were fixed in acetone at −20 °C for 15 min and air dried. Nonspecific binding sites were blocked by 20 min of incubation at 37 °C in RPMI 1640 medium supplemented with 10% FCS. The sections were then incubated with a 1:50 dilution of biotin-conjugated mouse anti-mouse PrP^c. After three washes in PBS, sections were incubated with a 1:300 dilution of Texas Red-streptavidin. Then the sections were washed and incubated with a 1:50 dilution of anti-mouse $I\text{-}A^{b,d,k}$ (clone BW/9, Seikagaku Co., Tokyo, Japan) at 37 °C for 30 min. Sections were washed and then incubated with a 1:100 dilution of FITC-goat anti-rat IgG at 37 °C for 30 min. After three washes in PBS, fluorescent samples were examined with a laser scanning confocal microscope.

2.8. Analysis of expression of cellular PrP^c mRNA

Cytoplasmic mRNA was isolated from 1×10^6 cells using QuickPrep Micro mRNA Purification Kits (Pharmacia Biotech, Uppsala, Sweden). cDNA was synthesized from mRNA using First-Strand cDNA Synthesis Kits (Pharmacia Biotech). The oligonucleotide primers used in this experiment were as follows: mouse PrP^c forward 5′-CTTTGTGACTATGTGG-3′, reverse 5′-GGCCTGCTCACGGCG-3′ [26], and G3PDH forward 5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′, reverse 5′-CATGTAGGCCATGAGGTCCACCAC-3′ [27]. The primers for mouse PrP^c and G3PDH were designed to generate fragments of 375 and 983 bp, respectively. Two-microliter aliquots of the cDNA reaction products were PCR amplified in 50-μl reactions containing the following: 5 μl of 10 × PCR buffer, each dNTP at 0.04 mM, 1 μl of each primer, 2.5 mM $MgCl_2$ and 0.5 μl of recombinant Taq DNA polymerase enzyme (Toyobo Co., Osaka, Japan). The reaction consisted of 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 2 min. The final extension was at 72 °C for 5 min. PCR products were analyzed by electrophoresis on 1.5% agarose gels and visualized by ethidium bromide staining.

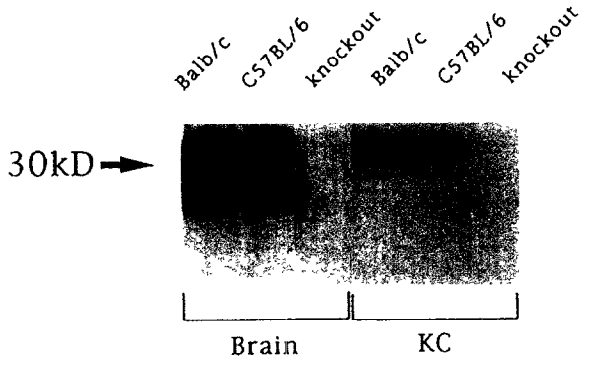

Fig. 1. Expression of PrP^c by brain and KC from BALB/c and from C57BL/6. Protein concentration in each sample was quantified by the Bradford method, and 50 μg protein was used for Western blot analysis. Nitrocellulose membranes were reacted with biotin-conjugated anti-mouse PrP^c followed by streptavidin-alkaline phosphatase.

3. Results

3.1. Expression of PrP^c by keratinocytes

Antibodies against-mouse PrP^c reacted with brain and KC from BALB/c mice and from C57BL/6 mice (wild type), but not from PrP^c knockout mice, by Western-blotting (Fig. 1). No visible protein band was detected using immunoglobulins from non-immunized control mice (data not shown). The reacted proteins consisted of a set of bands around 30 kD, which was consistent with the size range previously reported [8,28,29]. PrP^c was also detected on the cell surfaces of KC from BALB/c mice and from C57BL/6 mice (wild type), but not from PrP^c knockout mice, by FACS analysis (Fig. 2).

3.2. Keratinocytes and Langerhans cells expressed PrP^c in situ

KC of C57BL/6 mice (wild type) were positive for immunostaining of PrP^c in situ (Fig. 3a), while those of PrP^c knockout mice were negative (Fig. 3d). Basal and suprabasal KC up to the granular layer stained positive, while corneal layer was negative for PrP^c, which was the same with the previous reports [16,17]. I-A^b-positive dendritic cells in the epidermis, which are LC, were seen in both types of mice (Fig. 3b, e). Double-positive cells (yellow) were seen in epidermis of C57BL/6 mice (wild-type) (Fig. 3c), but not in the epidermis of PrP^c knockout mice (Fig. 3f). The staining patterns of BALB/c mice were the same as those of C57BL/6 mice (wild type). These data showed that murine KC and LC express PrP^c in situ.

3.3. Expression of PrP^c by Langerhans cells

Western-blotting showed that fLC from BALB/c mice expressed PrP^c, while those from PrP^c knockout mice did not (Fig. 4). The reacted protein showed a set of bands of MW around 30 kD, a range similar to that of the bands from KC.

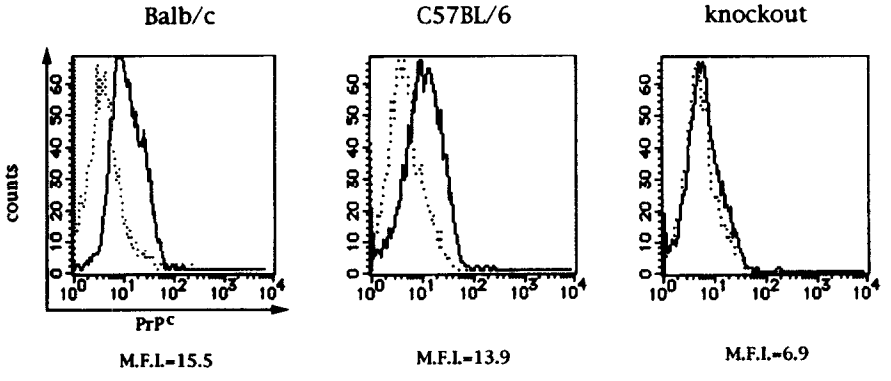

Fig. 2. FACS analysis showing PrP^c expression on KC from BALB/c and from C57BL/6. Cells were stained with biotin-conjugated anti-mouse PrP^c + FITC-streptavidin (solid lines). Biotin-conjugated mouse IgG + FITC-streptavidin was used as a negative control (dotted lines).

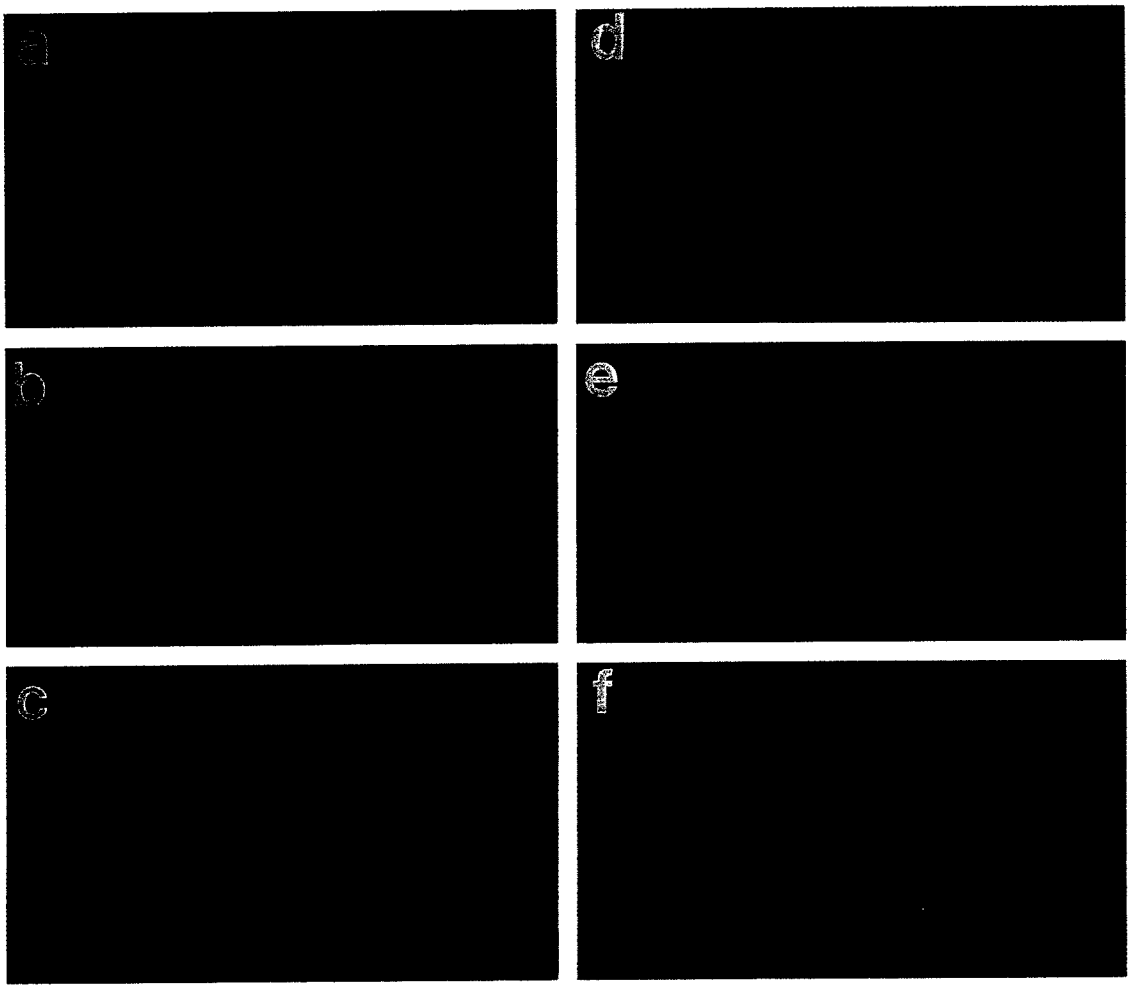

Fig. 3. Immunostaining of PrP^c in murine epidermis. Frozen sections were stained with biotin-conjugated mouse anti mouse PrP^c + Texas Red-streptavidin (a, d), then followed by rat anti-mouse I-A^b + FITC-goat anti-rat IgG (b, e). In composite photographs, double positive cells were colored yellow (c, f). (a-c): epidermis of C57BL/6 (wild type), (d–f): epidermis of PrP^c knockout mice.

Most fLC, which expressed I-A^d antigen, also expressed PrP^c as shown by FACS analysis (Fig. 5a). Both fLC and cLC expressed PrP^c, and the expression of PrP^c increased during culture (Fig. 5b).

3.4. Expression of PrP^c mRNA by keratinocytes and fLC

PrP^c mRNA was expressed both in fLC and KC from BALB/c mice (Fig. 6). KC from C57BL/6 mice (wild type) also contained PrP^c mRNA, while KC from PrP^c knockout mice did not.

4. Discussion

The results of this study demonstrated that murine LC and KC expressed PrP^c mRNA and protein and that LC retained the expression of PrP^c during culture.

Infection of prions is most effective when delivered directly to the brain of the host. This is not, however, the normal route of infection in the field. In humans, some cases of CJD transmission were traced to intramuscular injection of prion-contaminated pituitary hormones or dura grafting. Among animals, a more resent and fateful example is that of bovine spongiform encephalopathy (BSE), a common-source epidemic due to oral transmission of prions [1–3,6]. In addition, some experiments showed that scrapie infection occurred by the oral route via infection of the Peyer's patches followed by replication in the gut-associated lymphoid tissues [30]. In most cases of CJD and scrapie, however, the natural route of infection is totally unknown. Sporadic CJD can not attribute to oral transmission. Some epidemiological research has suggested that the infective agents may enter the body through breaks in the skin and mucous membranes [31] and some has suggested that the scrapie agent replicated in mites and that mites may represent a self-sustaining reservoir for scrapie-like agents [32]. In addition, successful transmission of prion diseases via inoculation into the skin has been reported in rodents [33]. Therefore, infection via skin can not be denied. Our data will give us the background to speculate that skin can be one of the target organs for the infective agents. As corneal layer of human and murine epidermis does not express PrP^c, infection through skin is possible only when there is some damage of corneal layer such as erosion or severe dermatitis.

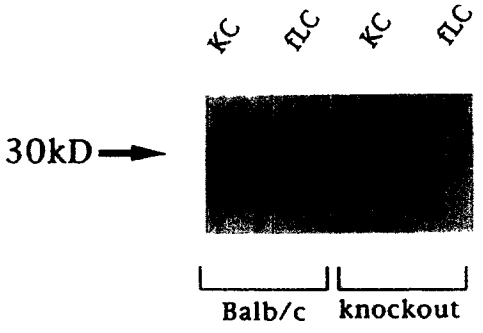

Fig. 4. Two hundred micrograms protein was used for Western blot analysis. Expression of PrP^c by fLC from BALB/c. Murine LC were enriched using the panning method.

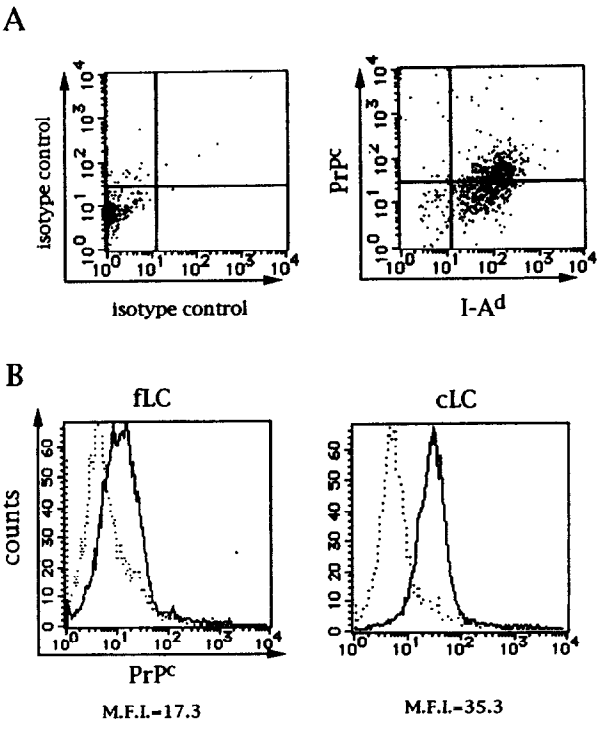

Fig. 5. Expression of PrP^c on the surface of fLC and cLC. (A) fLC were stained with biotin-conjugated anti-mouse PrP^c + PE-streptavidin and FITC-mouse anti-mouse $I\text{-}A^d$ (right), or controls (left). (B) fLC and cLC were stained with biotin-conjugated anti mouse PrP^c + FITC-streptavidin (solid lines). Biotin-conjugated mouse IgG + FITC-streptavidin was used as a negative control (dotted lines).

Many types of cells that have immunological functions, such as lymphocytes [9,14], macrophages [12], follicular dendritic cells (FDC) [11] and monocyte-derived dendritic cells [15] express PrP^c, although PrP^c expression is virtually absent on granulocytes [9,34,35]. These cells seem to play important roles in the pathogenesis of TSEs, not only because infectivity first appears in the LRS-tonsils, thymus, lymph nodes, and especially in spleen after introduction of the scrapie agent into various peripheral sites [36,37], but also because SCID mice did not develop neurological disease after peripheral inoculation of infectious

agents [4,6,30]. In particular, FDC have been shown to be the most important for the accumulation of PrP^{Sc} [4,11,38,39]. Using chimeric mice with a mismatch in PrP status between FDC and other cells of the immune system, it was reported that FDC, not lymphocytes or other bone marrow-derived cells, were involved in production and replication of PrP^{Sc} in spleen [38]. The role of other types of dendritic cells in different tissues, however, has not been fully investigated. Our data clearly showed that murine purified LC, myeloid dendritic cells (DC) in the skin [40] and upper digestive tract [41], express PrP^{c} mRNA and protein. Although the role of LC during the infection of prion diseases is still unknown, this study provides a possibility that myeloid DC might be important in prion diseases.

The expression of PrP^{c} has been proved to be controlled by cell type-specific regulatory mechanisms. In a human neural cell line (SK–N–SH cells), PrP^{c} mRNA was upregulated by TNF-α and IL-1β and was downregulated by IFN-γ [42]. PrP^{c} protein expression of human KC was shown to be upregulated by TGF-α and IFN-γ by Western blotting [16]. We examined the effects of cytokines (TNF-α, IL-1β, TGF-α and IFN-γ) on the expression of PrP^{c} on LC. PrP^{c} on the surface of murine LC was slightly upregulated by TNF-α and IFN-γ, but the changes were too small to be statistically significant (data not shown). The fact that LC retain PrP^{c} after culture and that cytokines do not diminish the expression of PrP^{c} on LC is quite important because it means that LC can maintain the expression of PrP^{c} during migration to lymph nodes. Although it is still unknown what kind of cells deliver infectivity (and PrP^{Sc}) to various organs, especially the LRS, after peripheral inoculation, our study will give us the background to speculate that LC can be one of the candidates.

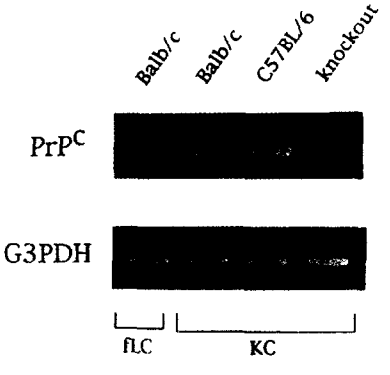

Fig. 6. Expression of PrP^{c} mRNA by fLC and KC from BALB/c and from C57BL/6. Cytoplasmic mRNA was isolated from 1×10^{6} fLC and from 1×10^{6} KC. PrP^{c} and G3PDH mRNA expression was examined by RT-PCR.

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